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LETTER TO THE EDITOR

The wax moth *Galleria mellonella* as a novel model system to study Enteroaggregative *Escherichia coli* pathogenesis

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Enteroaggregative *Escherichia coli* (EAEC) comprise of a large, diverse group of diarrheagenic *E. coli* defined by their characteristically 'stacked brick' pattern on HEp-2 cells.[1] EAEC was first associated with children's diarrhea in developing countries but studies have since shown that EAEC is the cause of both acute and persistent diarrhea in all ages worldwide.[2–6] Moreover, EAEC has been implicated in numerous outbreaks, most notably the large outbreak in Germany in 2011 with an EAEC strain lysogenized with a prophage harboring a *stx2a*-converting phage, resulting in 855 cases of hemolytic-uremic syndrome and 54 deaths.[7–11]

The pathogenesis of EAEC is not yet fully understood due to the heterogeneity among strains. EAEC are recognized as a diarrheal pathogen but are also isolated from healthy individuals stressing the need to be able to distinguish pathogenic from nonpathogenic EAEC strains. A variety of putative virulence factors have been identified, although none of these have been present only in the strains isolated from symptomatic patients.[12,13]

Even though the pathogenesis of EAEC is unclear, the 3 following stages have been suggested to occur upon infection; 1) initial adherence to the intestinal mucosa, possible by the aggregative adherence fimbriae (AAFs),[14,15] 2) biofilm formation[16] and 3) induction of an inflammatory response and the release of toxins.[16,17]

Understanding the complex relationship between the host and the bacterium is a crucial step for revealing the pathogenicity of a certain strain. Although many different animal models have been proposed for this pathogen, none have been able to show all of the clinical manifestation of disease.[18–21] Thus, there is still an urgent need for a reproducible animal model that is able to show all aspects of EAEC pathogenesis. Recently, larvae of the greater wax moth *Galleria mellonella* were established as an acceptable model to study bacterial infections caused by several pathogens including *Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas aeruginosa* and extra-intestinal E. coli.[22–25] The model has shown many advantages such as decreasing rearing costs, convenient feasibility, ability to carry out experiments at 37 °C and most importantly, correlation was observed between the *G. mellonella* model and well established vertebrate models.[26–28]

In this study, we sought to determine whether *G. mellonella* would be a suitable model for studying the virulence of EAEC infections.

The *G. mellonella* assays were performed as previously described by Morgan et al.[29] Briefly, bacterial overnight cultures were pelleted by centrifugation (4000 × g) and washed twice in PBS and finally re-suspended in PBS containing 10 % glucose. Groups of 10 larvae (200–250 mg) were infected with 10 μl aliquots of serially diluted bacterial suspensions (from 10^2 to 10^7 bacterial cells per larvae) by injection with a Hamilton syringe (26 gauge) via the last right proleg. Larvae were incubated at 37 °C after infection and survival was monitored for 96 hours.

Firstly, we investigated the virulence of 6 well characterized EAEC strains in the larvae model. One of the major challenges with EAEC is that the strains are highly heterogeneous with respect to genomic background, phylogroup, serotype, as well as their virulence genes. All strains tested harbored the 4 classical EAEC virulence factors: the transcriptional factor AggR, the aii island encoding a type VI secretion system, as well as dispersin and the dispersin transporter. Infection of *G. mellonella* with the EAEC strains resulted in rapid killing of the

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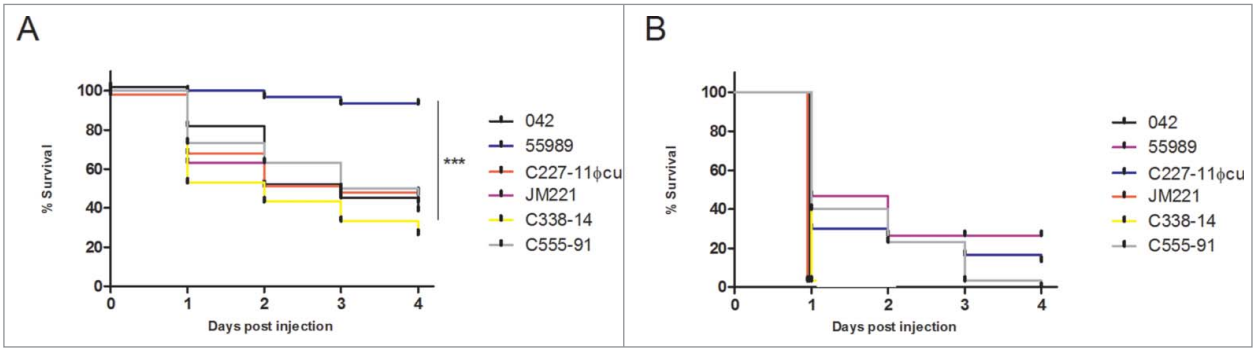

Figure 1. EAEC infection of *Galleria mellonella* larvae. Larvae were infected with 6 different EAEC strains and injected with different doses of bacteria; shown in the figures are injections of A) 10^3 CFU of EAEC injected into the larvae, where strain 55989 showed to be significantly different compared to the other 5 strains ($P < 0.001$) and B) 10^4 CFU of EAEC injected into the larvae. All results represents means of at least 3 independent experiments with 10 larvae per treatment. Survival curves were plotted using the Kaplan-Meier method and statistical analysis were performed using the log rank test for multiple comparisons (GraphPad Software, San Diego, CA).

larvae and mortality was shown to be dependent on the number of bacteria injected (Fig. 1AB). For all of the EAEC strains tested, 100 % of the larvae were killed after 24 hours with an inoculum of 10^6 CFU/larvae, whereas no mortality was observed when infected with 10^2 CFU/larvae (data not shown).

We next determined the LD50s after 24 hours of the strains as previously described.[30] Strain 042, the prototype EAEC strain which has also been shown experimentally to elicit diarrhea in human volunteers,[31] had the lowest LD50 of 1.11×10^4 CFU after 24 hours post infection. Compared to 042, the 5 other EAEC strains had similar LD50 values, except for strain 55989, which had a significantly higher LD50 compared to strain 042 ($P < 0.05$) (Fig. 2).

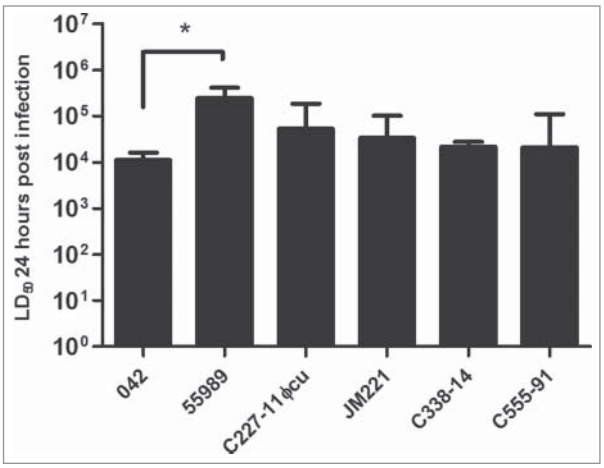

Figure 2. The LD50 of the strains were calculated using probit regression model (SPSS v. 20) for each isolate 24 h after *G. mellonella* larvae were inoculated with the bacteria. All strains showed to have similar LD50 value except for strain 55989, which had a significant higher LD50 than the other 5 strains tested ($P < 0.05$). Statistical significance between LD50 values were tested by performing one-way ANOVA with Dunnet post-test in GraphPad and the error bars displayed represent the 95 % confidence intervals.

It has previously been shown that nonpathogenic *E. coli* strain DH5α is not lethal to *G. mellonella* at inocula up to 10^7 CFU/larvae.[32] We repeated these experiments with 3 commensal *E. coli* strains: MG1655[33], HS [34] and F-18,[35] which confirmed previous data shown with DH5α. None of these commensal *E. coli* strains tested were lethal in the larvae up to 10^7 CFU (data not shown), indicating that it is the presence of EAEC virulence factors mediating the killing of the larvae. A recent study with *Caenorhabditis elegans* and infection with commensal *E. coli* strains have shown the O-antigens play an essential role in virulence in this model.[36] Thus, whereas the O-rough MG1655 wildtype showed a low killing of *C. elegans*, restoration of O-antigen expression enabled the strain to kill *C. elegans* at rates similar to EAEC strain 042. Furthermore, the commensal *E. coli* strain HS expressing the O9 antigen was as virulent as virulent in the *C. elegans* model as EAEC stain 042. In contrast, we found in the *G. mellonella* model that commensal strain HS was avirulent. Thus, whereas O-antigen expression may likely influence virulence in the *G. mellonella* model, high virulence in this model is not merely related to O-antigen expression.

This was also confirmed by injecting the larvae with the 6 heat-killed EAEC strains (10^7 CFU/mL). No killing was observed for any of the strains, suggesting that it is alive EAEC and not the LPS mediating the killing of the larvae (data not shown).

Next, we wanted to address whether the mortality of *G. mellonellla* is associated with the bacterial load of EAEC in the infected larvae. We therefore infected the larvae with 10^3 CFU of EAEC strain 042 and determined the bacterial load by enumeration of viable bacteria present at various time point. Each larvae were decapitated

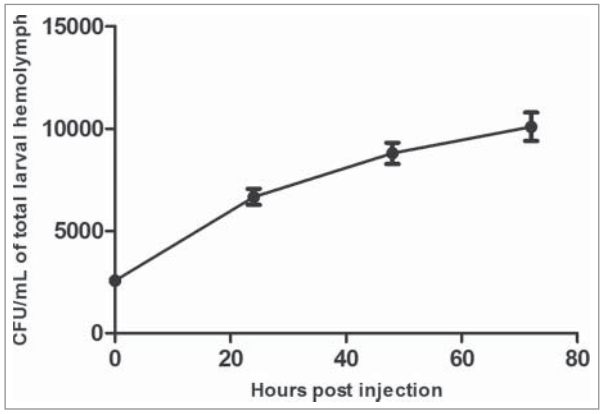

Figure 3. EAEC strain 042 grows inside the larvae. The larvae were infected with 10^3 CFU and at the indicated time points the hemolymph of 3 larvae were collected and CFU determined by plating on selective agar plates. Error bars at each indicated time point represents standard error of the mean.

and 30 μl of hemolymph were collected using a sterile 1.5 ml Eppendorf tube as previously described.[37] The hemolymph was serially diluted and plated on selective MacConkey agar plates containing chloramphenicol.[38] Infection with strain 042 resulted in an increase of bacteria over time in the hemolymph, demonstrating that 042 replicates inside the larvae (Fig. 3).

To evaluate whether a bacterial secretory product was involved in host death, a culture filtrate of strain 042 grown to stationary-phase at 20 and 37 °C in LB-broth or the cell medium DMEM/0.5% glucose which have previously shown to upregulate EAEC virulence genes.[39] The supernatants were concentrated 10 times using a 10 kDa amicon filter (Merck Millipore, Kenilworth, NJ) and inoculated into the larvae. The results showed that a

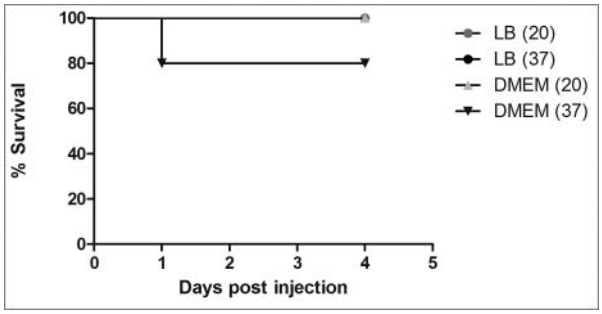

Figure 4. Supernatant of EAEC strain 042 has little effect on mortality. Strain 042 was grown stationary overnight in either LB-broth or DMEM/0.5 % glucose at 20 and 37 °C. The supernatants were then sterile filtered and concentrated 10 times using an 10 kDa amicon filter (Merck Millipore, Kenilworth, NJ), and 10 μl were injected into the larvae. The results represents the average of 3 experiments, repeated with 3 different batches of larvae. Statistical difference was calculated using the log rank test for multiple comparisons in GraphPad, however no statistical significance was observed.

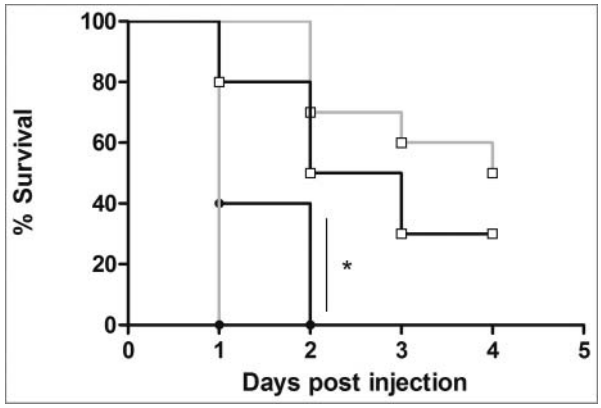

Figure 5. Strain 042 shows temperature dependent mortality. *G. mellonella* strains were infected with strain 042 (□10^3 and •10^4) and incubated at either 22 °C (blue) or 37 °C (black) for 4 d Statistical analysis were performed using the log rank test for multiple comparisons (GraphPad), and a significant difference was observed between larvae inoculated with 10^4 CFU and the 2 temperatures used ($P < 0.05$).

small fraction of larvae were killed when grown in DMEM/0.5 % glucose at 37 °C, whereas no killing was observed at 20 °C in DMEM/0.5 % glucose or when grown in LB-broth (Fig. 4).

Lastly, we investigated whether the larvae incubation temperature changed the killing rate of larvae by EAEC strain 042. Larvae were injected as previously described and incubated at either 22 °C or 37 °C and mortality was recorded for up to 4 d From these experiments, we could conclude that the lowered temperature attenuated the killing of the larvae (Fig. 5). For example, at day 1 40 % of the larvae were still alive at room temperature with a dose of 10^4 CFU/larva whereas none of the larvae incubated at 37 °C were alive ($P < 0.05$). These data suggest that 042 has temperature sensitive virulence traits. This could be speculated to be related to a lower expression of the key regulator of EAEC virulence factors, AggR, which has been shown to be optimally expressed at 37 °C.[39]

In conclusion, we report that EAEC is able to infect and kill *G. mellonella* in a dose and time dependent manner, and that the model is able to distinguish clearly between virulent wildtype strains of EAEC and non-pathogenic *E. coli*. Moreover, we also see that EAEC is able to survive and replicate in the larvae, and that viable EAEC are needed to cause mortality. Previous infection studies using ExPEC in the *G. mellonella* model showed that with doses below 5×10^6 CFU, less than 30 % of larvae were killed after 4 d whereas when using a dose above 5×10^6 CFU, over 80 % of the larvae were killed within 24 h.[40] We here show that a dose of 1×10^6 is sufficient for 100 % mortality within 24 h with the EAEC strain 042, suggesting that EAEC could be an even more virulent pathotype than ExPEC. Interestingly, a study investigating ExPEC and virulence factors found the

Afa/Dr adhesins to be associated with significantly higher mortality, suggesting that the fimbriae is important in ExPEC pathogenicity.[41] The Afa/Dr adhesins are very homologous to the AAF fimbriae encoded by EAEC, and it could be speculated that AAF fimbriae plays a role in the high virulence of EAEC in the larvae.

However, many questions remains open, for example which virulence factors are mediating the killing of the larvae and is it one or a combination of multiple? To address these questions, it will be necessary to investigate a wide array of mutants from various strains, since EAEC is so heterogeneous and one virulence factor may be important in one strain but less important in another. That the *G. mellonella* model can be a valuable tool to future studies of EAEC pathogenicity is supported by the successful use of this model to study enteropathogenic *E. coli* (EPEC) virulence.[42] However, the model does not replace well-established mammalian models, but it is an inexpensive and reliable model providing the ability to study the difference between virulent and non-virulent EAEC strains, identification of putative virulence markers, and possible novel molecular targets for antimicrobial therapy and vaccine development.

Disclosure of potential conflicts of interest

The authors have no conflict of interest to declare

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